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Angiogenesis, the formation of capillaries from pre-existing blood vessels, is essential for sustained growth of solid tumors. Numerous studies have shown that copper is required to modulate several pro-angiogenic factors. However, the specific effects of copper homeostasis on tumor angiogenesis have not been extensively studied. Our preliminary studies demonstrated that tetrathiomolybdate, a potent and novel copper chelator, blocks tumor growth and angiogenesis. We hypothesize that TM is inhibiting tumor angiogenesis by decreasing levels of VEGF, bFGF, IL-6, and IL-8 through interference with the NFkB signaling cascade. In this proposal, the molecular mechanism whereby TM regulates NFkB expression and activity will be investigated. We will establish if the NFkB transcription factor complexes, p50, p52, RelA, and RelB, are regulated by TM using Western blot analysis and gel shift assays. Furthermore, using a reporter gene system, we will ascertain if TM regulation of VEGF, bFGF, IL-6, and IL-8 is a direct consequence of NFkB signal inhibition. The studies as outlined will help us better understand the role of copper deficiency in tumor angiogenesis and may lead to a more specific and potent global anti-angiogenic approach to treat breast cancer.

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Quintin Pan, Ph.D.
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August 22, 2003

Introduction:

The NFκB/Rel family of transcription factors is comprised of RelA, RelB, c-Rel, p50 (nfκb1), and p52 (nfκb2) (1). In recent years, evidence linking uncontrolled NFκB activity to oncogenesis has emerged. NFκB has been shown to regulate genes important for invasion, angiogenesis, and metastasis. These include pro-angiogenic factors, such as VEGF, IL-6, and IL-8, matrix metalloproteinases, urokinase plasminogen activator (uPA), and cell adhesion molecules, such as ICAM-1 and VCAM-1 (2-6). Blocking NFκB activity in human ovarian cancer cells was shown to inhibit VEGF and IL-8 expression resulting in a decrease in tumor angiogenesis (7). Using SUM149 human inflammatory breast cancer cells, we demonstrate that p50 (nfκb1) and RelA protein levels are decreased in response to TM. Our preliminary data indicate that TM also was able to block NFκB-dependent transcription in these cells. Moreover, apoptosis was increased 2-fold in SUM149 cells following TM treatment. Taken together, our data lead us to hypothesize that TM is blocking tumor angiogenesis by decreasing levels of pro-angiogenic mediators, VEGF, bFGF, IL-6, and IL-8, and inducing apoptosis through interference with the NFκB signaling cascade. The specific aims as outlined in this proposal will help us to better understand the role of copper deficiency in tumor angiogenesis and apoptosis and may lead to a more specific approach to treat breast and other cancers.

Body:

Task 1: To determine the molecular mechanism whereby TM regulates NFkB expression and activity in human breast cancers (Months 1-12).

We determined whether copper deficiency induced by TM is modulating NFκB-mediated signaling. SUM149 and non-tumorigenic immortalized human mammary epithelial (HME) cells were transiently transfected with pNFκB, a vector that contains four tandem copies of the κB consensus sequence upstream of the luciferase reporter gene. Endogenous NFκB activity was shown to be 2.5-fold higher in SUM149 cells in comparison to HME cells. This is consistent with our observation that p50 protein levels were significantly higher in SUM149 cells (data not shown). After treatment for 24 hours, TM inhibited luciferase activity by 62±2% (P<0.001, n=6) in SUM149 cells and 34±2% (P<0.001, n=3) in HME cells (Figure 1). Moreover, TM completely blocked TNFα-stimulated NFκB activity in both cell lines. Similar results were observed at 48 and 72 hours demonstrating that TM is also able to inhibit NFκB activity on a sustained basis without affecting cell survival under these conditions. As shown in Figure 2, we analyzed the binding of nuclear proteins from SUM149 cells to a labeled oligonucleotide spanning the κB consensus sequence. Extracts from TM-treated cells showed a decrease in nuclear protein binding to the κB consensus sequence. In addition, supershift analysis revealed that the predominant NFκB components in SUM149 cells are p50, p52, and RelA. When cells were cultured with added copper (2 nM $CuSO_4$ addition), TM partially

lost its ability to regulate κB binding and NFκB transcriptional activity. Also, copper repletion partially reversed TM-inhibition of IL-6 and IL-8 mRNA expression, consistent with restoring NFκB's ability to enhance transcription of these genes. Interestingly, p50 and RelA protein levels were reduced following treatment with TM in SUM149 cells suggesting that TM may be suppressing NFκB activity by decreasing levels of NFκB component proteins.

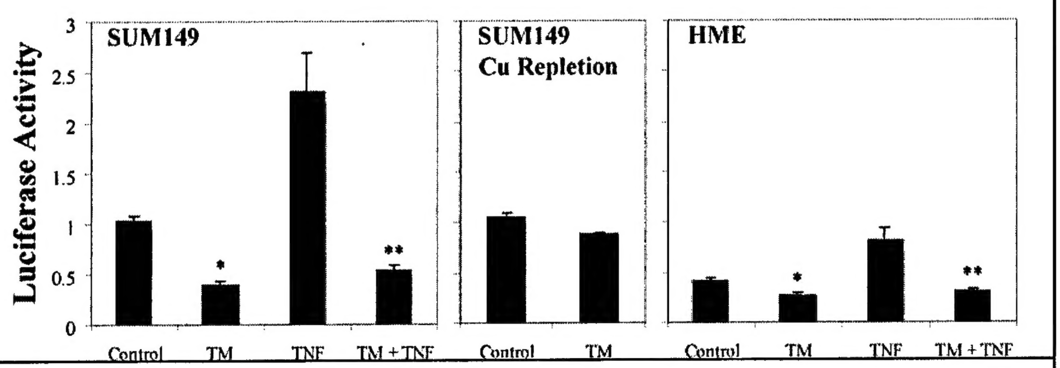

Figure 1. TM Suppresses NFκB-dependent Transcription. SUM149 or HME cells were transfected with pNFκB (Clontech Laboratories Inc.), a vector that contains four tandem copies of the κB consensus sequence fused to a TATA-like promoter region from the Herpes simplex virus thymidine kinase promoter. pRL-TK (Promega Corp.), a *Renilla* luciferase vector, was co-transfected into the cells to normalize for transfection efficiency. After a 24 hour recovery period, transfected cells were incubated in fresh medium with or without the addition of 2 nM $CuSO_4$. Cell were subsequently treated with vehicle, TM (1 nM), TNFα (2 pM), or TM and TNFα for 24 hours. *$P<0.001$; $n=6$, vehicle vs. TM. **$P<0.001$; $n=6$, vehicle or TNFα vs. TM + TNFα.

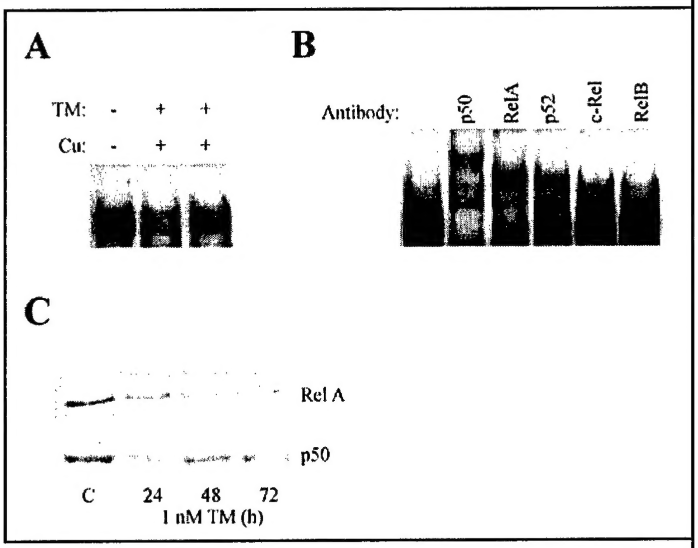

Figure 2. TM Decreases NFκB Binding and Protein Levels. **A,** Gel Shift Analysis. SUM149 cells were treated with vehicle or TM (1 nM) with or without the addition of 2nM $CuSO_4$ for 72 hours. Nucelar proteins were extracted, incubated with ^{32}P-labeled κB consensus sequence, and resolved by EMSA. **B,** Identification of NFκB Binding Complex. Supershift analysis was performed by pre-incubating nuclear extracts with p50, RelA, p52, c-Rel, or RelB antibody (Upstate Biotechnology) for 20 minutes on ice. **C,** p50 and RelA Protein Levels. SUM149 cells were treated with vehicle or TM (1 nM) for 72 hours. p50 and RelA levels were determined by Western blot analysis. Data is representative of three independent experiments.

Task 2: To determine if TM regulation of VEGF, bFGF, IL-6, and IL-8 is a direct consequence of inhibiting NFκB activity (Months 13-30).

We characterized the effect of NFκB suppression in SUM149 cells by genetically inhibiting NFκB with a dominant negative IκBα (S32AS36A). As shown in Figure 3, conditioned-media from SUM149 wildtype and SUM149-IκBαMut clones were collected and measured for VEGF, IL-1α, and IL-8 by ELISA. Secretion of these proangiogenic mediators by SUM149 wildtype cells cells was significantly inhibited following TM treatment (10 nM for 72 hours). Similarly, SUM149-IκBαMut clones 2 and 3 secreted lower amounts of these proangiogenic mediators in comparison to untransfected or empty-vector transfected SUM149 cells ($p<0.05$) (Figure 2A). TM treatment of SUM149-IκBαMut clones resulted in no additional effect on inhibiting the amount of proangiogenic mediators. Since the change in phenotype observed for TM-treated SUM149 cells are similar to untreated SUM149-IκBαMut clones and TM did not change the phenotype of SUM149-IκBαMut clones, there is evidence that inhibition of tumor angiogenesis by TM is a direct consequence of TM's ability to suppress NFκB activation.

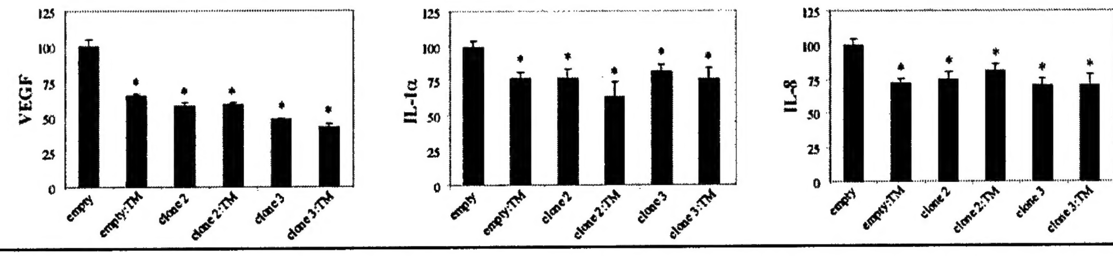

Figure 3. Characterization of proangiogenic mediators in SUM149 and SUM149-IκBαMut clones. SUM149, SUM149-empty, SUM149-IκBαMut clone2, or SUM149-IκBαMut clone3 cells were plated and treated with vehicle or 10 nM TM for 72 hours. Conditioned-media was collected and measured for VEGF, IL-1α and IL-8 by ELISA. *, $p<0.05$ vs. untreated SUM149-empty vector.

Key Research Accomplishments and Reportable Outcomes:

1. TM was found to significantly decrease NFκB protein levels and transcriptional activity.

2. TM was found to decrease the levels of potent proangiogenic mediators, VEGF, IL-1α, and IL-8.

3. We identified a major mechanism of the antiangiogenic effect of copper deficiency induced by TM is suppression of NFκB, contributing to a global inhibition of NFκB-mediated transcription of proangiogenic mediators.

Conclusions:

We have made significant progress in the past year in understanding how TM acts as an anti-angiogenic compound. TM was found to be an indirect anti-angiogenic by inhibiting NFκB activity of tumor cells resulting in decreased secretion of NFκB-dependent pro-angiogenic factor.

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